DCIR Acts as an Inhibitory Receptor Depending on its Immunoreceptor Tyrosine-Based Inhibitory Motif[1]

Nobuo Kanazawa,*† Taku Okazaki,† Hiroyuki Nishimura,† Kei Tashiro,‡ Kayo Inaba,§ and Yoshiki Miyachi*

*Department of Dermatology and †Medical Chemistry, Graduate School of Medicine, ‡Center for Molecular Biology and Genetics, and §Laboratory of Immunobiology, Division of Systemic Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Major histocompatibility complex class II positive cells, namely dendritic cells, monocytes/macrophages, and B cells, are categorized as antigen-presenting cells. Dendritic cells, so-called professional antigen-presenting cells, use distinct sets of surface receptors before and after maturation: those to capture antigens and those to interact with T cells, respectively. But there remain many surface molecules whose functions are still unknown. In this study, we isolated dendritic cell immunoreceptor from mouse bone-marrow-derived mature dendritic cells. Dendritic cell immunoreceptor is a recently reported C-type lectin receptor characteristic with cytoplasmic immunoreceptor tyrosine-based inhibitory motif. Expression of mouse dendritic cell immunoreceptor mRNA was observed specifically in spleen and lymph node, slightly increased with dendritic cell maturation during *in vitro* culture of bone marrow cells, and was not detected in cultured natural killer cells. Surface expression of mouse dendritic cell immunoreceptor protein was observed in splenic antigen-presenting cells including B cells, monocytes/macrophages, and dendritic cells, but not in T cells. To reveal the downregulating capacity of dendritic cell immunoreceptor in antigen-presenting cells, the change of B-cell-receptor-mediated signals after coligation with a chimeric Fcγ receptor IIB containing the cytoplasmic portion of mouse dendritic cell immunoreceptor was examined. As a result, we detected two distinct inhibitory effects of cytoplasmic dendritic cell immunoreceptor – inhibition of B-cell-receptor-mediated Ca^{2+} mobilization and protein tyrosine phosphorylation – and both of these effects required the tyrosine residue inside the immunoreceptor tyrosine-based inhibitory motif. This report presents immunoreceptor tyrosine-based inhibitory motif-dependent negative regulatory function of dendritic cell immunoreceptors. In conclusion, mouse dendritic cell immunoreceptor expressed on antigen-presenting cells can exert two distinct inhibitory signals depending on its immunoreceptor tyrosine-based inhibitory motif tyrosine residue. ***Key words: antigen-presenting cells/C-type lectin/DCIR/dendritic cells/ITIM. J Invest Dermatol 118:261–266, 2002***

Major histocompatibility complex (MHC) class II positive cells, namely dendritic cells (DC), monocytes/macrophages, and B cells, are categorized as antigen-presenting cells (APC). Among them, DC form a system of bone-marrow-derived "professional" APC specialized in interaction with T cells (Banchereau and Steinman, 1998). Immature DC are localized in peripheral tissues and function as sentinels. Once DC capture antigens, they migrate into T cell areas of draining lymphoid organs, become mature, and there present processed antigenic peptides to T cells. Many surface receptors play distinct roles in each aspect of DC functions. Among them, C-type (Ca^{2+}-dependent) lectin family receptors are considered to recognize carbohydrate-bearing molecules using their carbohydrate recognition domain (CRD). Multiple (8–10) CRD-containing type I membrane receptors, such as macrophage-mannose receptor (Stahl and Ezekowitz, 1998) and DEC-205 (Jiang *et al*, 1995), are reported to work in internalization of carbohydrate-bearing antigens. In contrast various single CRD-containing type II membrane receptors, most of which have been identified in recent years, are likely to have more complicated functions: langerin works in antigen uptake (Valladeau *et al*, 2000); DC-SIGN acts as an adhesion molecule (Geijtenbeek *et al*, 2000); and dectin-1 has a costimulatory capacity (Ariizumi *et al*, 2000b).

The recently reported single CRD-containing type II receptor, DC immunoreceptor (DCIR) (Bates *et al*, 1999), is distinct from others by its cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) (Vivier and Daeron, 1997; Bolland and Ravetch, 1999). ITIM-bearing inhibitory receptors include two subgroups, the Ig superfamily and the C-type lectin family. DCIR is the first

Manuscript received June 13, 2001; revised July 24, 2001; accepted for publication September 3, 2001.

Reprint requests to: Dr. Kei Tashiro, Center for Molecular Biology and Genetics, Kyoto University, Shogo-in kawaharacho 53, Sakyo-ku, Kyoto 606-8507, Japan. Email: ktashiro@mfour.med.kyoto-u.ac.jp

Abbreviations: BCR, B cell receptor; BM-DC, bone-marrow-derived dendritic cells; CRD, carbohydrate recognition domain; DCIR, dendritic cell immunoreceptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; PY, phosphotyrosine; SST, signal sequence trap.

[1]A preliminary report of these results was presented by the first author at the 61st annual meeting of SID in Chicago in the session "General Immunology".

reported DC-expressing ITIM-bearing receptor of the C-type lectin family. Its inhibitory function has been undetected so far, however.

Here we describe the isolation and expression profile of mouse DCIR and, furthermore, successfully show that its cytoplasmic ITIM sequence does indeed deliver two distinct negative regulatory signals in APC.

MATERIALS AND METHODS

Cells and mice Mouse bone-marrow-derived DC (BM-DC) were prepared as described previously (Inaba *et al*, 1992). In brief, BALB/C mouse bone marrow cells were cultured in the presence of recombinant mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) and at day 6 the clusters of developing DC were transferred to a new plate and recultured to allow maturation. In some experiments, 0.5 ng per ml recombinant human transforming growth factor (TGF) β1 (R&D Systems, Minneapolis, MN) was added to the DC culture after replating at day 6. In order to prepare natural killer (NK) cells, C57BL/6 mouse bone marrow cells were cultured in the presence of recombinant human interleukin-15 (IL-15) (PeproTech EC, London, U.K.) as described previously (Ogasawara *et al*, 1998). Collagenase (Wako Pure Chemical Industries, Osaka, Japan) treated C57BL/6 mouse splenic cells were prepared as described previously (Crowley *et al*, 1989). Flow cytometry (FCM) analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ). Some mice were intraperitoneally immunized with 1×10^8 sheep red blood cells (Cosmo Bio, Tokyo, Japan).

Nucleic acid analysis Construction of the cDNA library and screening to isolate secretory pathway proteins using the signal sequence trap (SST) method were performed as described previously (Jacobs *et al*, 1997). In brief, 5′-enriched random-primed cDNA fragments were size-fractionated and, after amplification by polymerase chain reaction (PCR), cloned into an expression vector bearing invertase cDNA without signal sequence as a reporter. The library was introduced into *SUC2*$^-$ yeast cells that could not survive without secretable invertase on the selection plate and, after 3–7 d selection, surviving colonies were picked up and base sequences of their plasmids were determined. After BLAST and FASTA search, IMAGE consortium cDNA clones containing identical expression-sequence tags were purchased and their base sequences were determined. The cDNA fragment of DCIR (nucleotides 272–577) labeled with [α-^{32}P]dCTP was used as a probe for northern hybridization. Reverse transcription (RT)-PCR was performed with the following primer pairs: for DCIR, 5′-GAT CTA AGA AAG CCT GGT TC-3′, 5′-GCA AGA GAT ATC GTT CCA GC-3′; for NKR-P1A, 5′-TGA TGC ATC TCC TAT GCA C-3′, 5′-CAA TCT GTT GTT TTA TTC AGG-3′; for NKR-P1B, 5′-GTC AAG TCC CTC CAT CTA C-3′, 5′-CAA TCT GTT GTA TGT GTT CTG-3′; for NKR-P1C: 5′-CTT CTC ACC ACC AGT TAA G-3′, 5′-GGC ACT CTA AAT TAA CTG TTG-3′. The conditions adapted for the Perkin-Elmer 9700 were described previously (Kanazawa *et al*, 1999).

Transformants The PCR-amplified cDNA fragment of fractalkine signal sequence (nucleotides 77–160 of GenBank AF071549) combined with that of N-terminal histidine-tagged (six amino acids: HHHHHH) mouse DCIR extracellular domain (nucleotides 485–991) for the soluble form of DCIR, or that of full-length mouse DCIR (nucleotides 278–991) with C-terminal FLAG epitope (eight amino acids: DYKDDDDK) for membranous DCIR, was cloned into pCMX-PL1 expression vector (Umesono *et al*, 1991) and transiently introduced into 293T cells (Numa *et al*, 1995) using CellPhect Transfection Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR-amplified cDNA fragment of cytoplasmic mouse DCIR (nucleotides 278–508), its $^{\Delta}$Y mutant (nucleotide 297 A to T) or cytoplasmic CD72 (nucleotides 33–410 of GenBank S40777), which were combined with that of C-terminal FLAG-tagged extracellular Fcγ receptor IIB (FcγRIIB; nucleotides 94–612 of GenBank U51629), was cloned into retroviral pMX-IRES-EGFP vector (Kitamura, 1998) and permanently introduced into IIA1.6 cells (Jones *et al*, 1986) as described elsewhere.[2] Green fluorescent protein (GFP)-positive transformants were sorted using FACSVantage (Becton Dickinson). Empty vector DNA was used as a negative control.

[2]Okazaki T, Macda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting scr homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci, USA* (in press).

Antibodies Rabbit polyclonal antibodies were raised against two polypeptides corresponding to the extracellular portions of mouse DCIR, GHRQWQWVDQTPYEES and QSQEEQDFITGILDTH, and affinity-purified with each peptide (Sawady Technologies, Tokyo, Japan). Normal rabbit Ig (Dako Japan, Kyoto, Japan) was used as a negative control. Horseradish peroxidase (HRP) conjugated anti-6xHis (Clontech Laboratories, Palo Alto, CA), biotinylated anti-FLAG M2 (Sigma Chemical, St. Louis, MO), intact or F(ab′)$_2$ fragment of rabbit antimouse IgG (H + L) (Zymed, San Francisco, CA), and agarose or HRP-conjugated antiphosphotyrosine (anti-PY) antibodies (Transduction Laboratories, Lexington, KY) were purchased. Fluorescein isothiocyanate (FITC) conjugated anti-CD3ε, phycoerythrin (PE) conjugated anti-NK1.1, B220, CD19, Mac1α, CD3ε, I-A/I-E, or γδ T cell receptor (TCR), and biotinylated anti-CD11c, B7.1, or B7.2 antibodies were purchased from PharMingen (San Diego, CA). As the secondary antibodies, HRP-conjugated donkey antirabbit IgG (Amersham Pharmacia Biotech), Texas Red (Vector Laboratories, Burlingame, CA), or FITC-conjugated goat antirabbit IgG (H + L) antibodies (Jackson Immunoresearch Laboratories, West Grove, PA), and FITC- or PE-conjugated streptavidin (PharMingen) were used.

Calcium mobilization assay IIA1.6 transformants (8×10^6 cells per ml) were loaded with 10 μM fura-red (Molecular Probes, Eugene, OR) in 10 mM HEPES-containing medium at 37°C for 30 min, inverted frequently. Collected cells were resuspended in the same medium (1.6×10^6 cells per ml). After addition of 10 μg per ml antimouse IgG, the cells were analyzed by FCM.

Immunoprecipitation IIA1.6 transformants (1.5×10^7 cells per ml) were incubated with 25 μg per ml antimouse IgG at 37°C for 1 min until addition of ice-cold phosphate-buffered saline. Preparation of cell lysates was performed as described previously.[2] Precleared lysates were incubated with agarose-conjugated anti-PY at 4°C for 2 h. Immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and further provided for Western blotting as described previously (Yabe *et al*, 1997).

RESULTS

Mouse DCIR is isolated from *in vitro* differentiated DC In order to isolate soluble factors and their receptors expressed in DC, we screened cDNA libraries constructed from TGF-β1 responsive mouse BM-DC after 8 d culture using the SST method. Starting from 10^6 independent cDNA clones, we picked up 520 surviving yeast colonies, eliminated artifact clones, and finally obtained 24 independent genes. Known genes and counterparts of reported genes in other species isolated by this study are listed in **Table I**. Among them, we selected a clone that is identical to recently discovered DCIR (C-type lectin, superfamily member 6; Clecsf6) for further investigation and confirmed its full-length cDNA sequence (the nucleic acid sequence that we determined for mouse DCIR has been submitted to GenBank under accession number AF387099). Mouse DCIR is a type II membrane C-type lectin receptor and the amino acid sequence of its CRD is the most homologous to that of dectin-2, the recently identified DC-specific lectin (Ariizumi *et al*, 2000a), showing 48% identity, in contrast with 55% between mouse and human DCIR. Furthermore, there exist some other type II C-type lectins that are also highly homologous to mouse DCIR and preferentially expressed in DC and/or macrophages, such as macrophage-inducible C-type lectin (42% identity; Matsumoto *et al*, 1999), macrophage-restricted C-type lectin (38%; Balch *et al*, 1998), and macrophage calcium-type lectin (33%; Mizuochi *et al*, 1997), as well as human DC-SIGN (36%) and DC-SIGNR (37%; Soilleux *et al*, 2000). Among these lectins, only DCIR bears cytoplasmic ITIM and has been predicted to have a distinct inhibitory function (Bates *et al*, 1999).

Mouse DCIR is expressed on cultured mature DC but not on NK cells Among normal tissues, mouse DCIR is specifically expressed in spleen and lymph node, both tissues being rich in DC (**Fig 1*a***). Furthermore, the change of mouse DCIR expression level during bone-marrow-derived DC differentiation was examined by northern hybridization. As shown in **Fig 1(*b*)**, although DCIR expression was very faint in fresh bone marrow cells, it became detectable after 4 d culture with GM-CSF, slightly

Table I. List of secretion pathway proteins isolated by the yeast-SST from mouse TGF-β1 responsive BM-DC library.

Accesion no.	Protein
Known	
AB021289	epididymal secertory protein (ME1)
AF290973	lysosomal thiol reductase IP30
L20315	MPS1
M30655	phagocytic glycoprotein-1 (Pgp-1, CD44)
NM_008401	integrin alpha M (Itgam, CD11b)
NM_008526	lyphocyte antigen 55 complex, locus B (Ly55b, NKR-P1B)
NM_009137	small inducible cytokine subfamily A, member 22 (Scya22, MDC)
NM_009263	secreted phosphoprotein 1 (Spp1, osteopontin)
NM_009976	cystatin C (Cst3)
NM_010299	GM2 ganglioside activator protein (Gm2a)
NM_010378	histocompatibility 2, class II antigen A, alpha (H2-Aalpha)
NM_011113	urokinase plasminogen activator response (uPAR)
NM_011999	C-type lectin, superfamily member 6 (Clecsf6, DCIR)
NM_013532	glycoprotein 49 B (Gp49b)
NM_016898	CD164 antigen (MGC-24)
NM_017372	lysozyme (Lyzs)
NM_021334	integrin alpha X (Itgax, CD11c)
Counterparts	
AB029028	human KIAA1105 protein
NM_006495	human ecotropic viral integration site 2B (EVI2B)
NM_014742	human KIAA0255 gene product

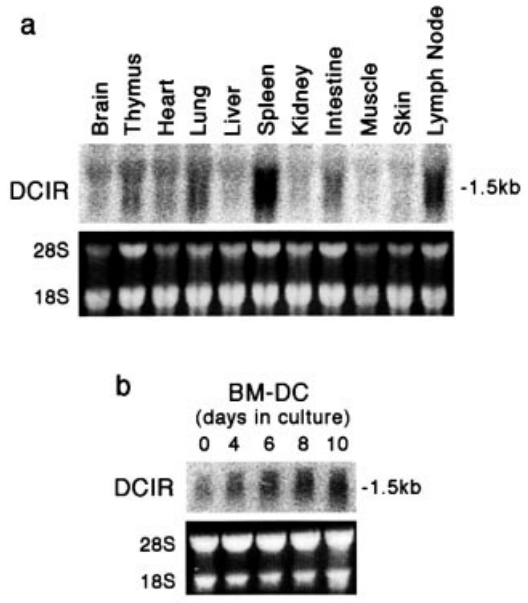

Figure 1. Mouse DCIR expression among normal tissues and maturating DC. Total RNA was isolated from mouse normal tissues (*a*) and bone-marrow-derived GM-CSF responsive DC harvested at day 0, 4, 6, 8, and 10 (*b*). DCIR mRNA level was determined by northern hybridization. 18 μg total RNA was applied for each lane.

increased with time, and reached a maximum on day 10. These data suggest that mouse DCIR expression slightly increases with DC maturation.

As the domain structure of DCIR, composed of cytoplasmic ITIM and extracellular C-type lectin, was similar to that of NK cell inhibitory receptors and the human DCIR gene is located close to the NK gene complex (Bates *et al*, 1999), mouse DCIR expression

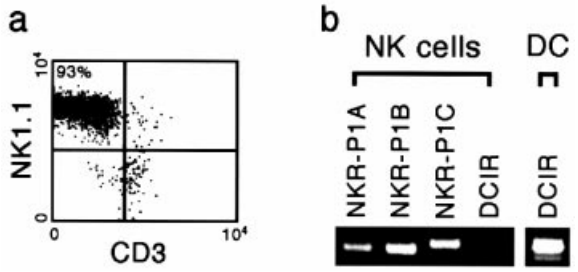

Figure 2. No expression of mouse DCIR in bone-marrow-derived NK cells. (*a*) Flow diagram of CD3 and NK1.1 expression on bone-marrow-derived NK cells cultured with IL-15 for 10 d. (*b*) Total RNA (600 ng) from bone-marrow-derived NK cells and DC harvested at days 13 and 8, respectively, was reverse transcribed using oligo-dT primer. PCR was performed using specific primer pairs for NK cell receptors or DCIR.

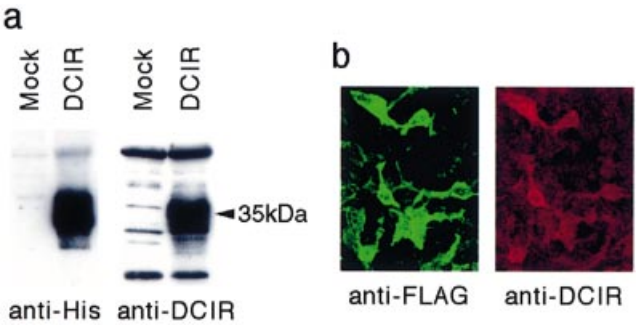

Figure 3. Specificity of anti-DCIR antibodies. (*a*) Serum-free culture supernatant of 293T cells transfected with the histidine-tagged soluble form of DCIR was analyzed by Western blotting using anti-6xHis (*left column*) or anti-DCIR antibodies (*right column*). (*b*) 293T cells expressing FLAG-tagged full DCIR were stained with anti-FLAG (*left column*) or anti-DCIR antibodies (*right column*) without permealyzation.

in NK cells was examined by RT-PCR. As shown in **Fig 2**(***a***), after 10 d culture with IL-15, more than 90% of C57BL/6 mouse bone marrow cells represent $CD3^-$ $NK1.1^+$ NK cells. Although a DCIR-specific band was detected in BM-DC, no expression of DCIR was detected in these NK cells harvested on day 13, in contrast to the strong expression of NK cell receptors such as NKR-P1A, NKR-P1B, and NKR-P1C (**Fig 2*b***).

Mouse DCIR protein is expressed on the surface of splenic APC To observe the protein expression of mouse DCIR *in vivo*, anti-DCIR polyclonal antibodies were raised and collagenase-treated mouse splenocytes were analyzed by two-color FCM. Specificity of anti-DCIR antibodies was confirmed by Western blotting of the culture supernatant of 293T cells transfected with the soluble form of DCIR (**Fig 3*a***), by surface immunostaining of 293T cells transfected with membranous DCIR (**Fig 3*b***), and furthermore by FCM analysis of mouse splenocytes (**Fig 4*a***). As shown in **Fig 4**(***b***), almost all the $B220^+$ or $CD19^+$ B cells, $Mac1^+$ monocytes/macrophages, and $CD11c^+$ DC expressed DCIR, whereas, in contrast, $CD3^+$ T cells did not. It should be noted that MHC class II was well coexpressed with DCIR (**Fig 4*b***), indicating that DCIR is unexceptionally expressed on the surface of MHC class II^+ APC including B cells, monocytes/macrophages, and DC. $B7.1^+$ or $B7.2^+$ activated cells also expressed DCIR and, after immunization, DCIR expression still remained high, in accordance with the induction of B7.1 or B7.2 expression (**Fig 4*c***). Two independent antibodies specific for different portions of DCIR gave the same results and representative results obtained using antibodies against GHRQWQWVDQTPYEES are shown.

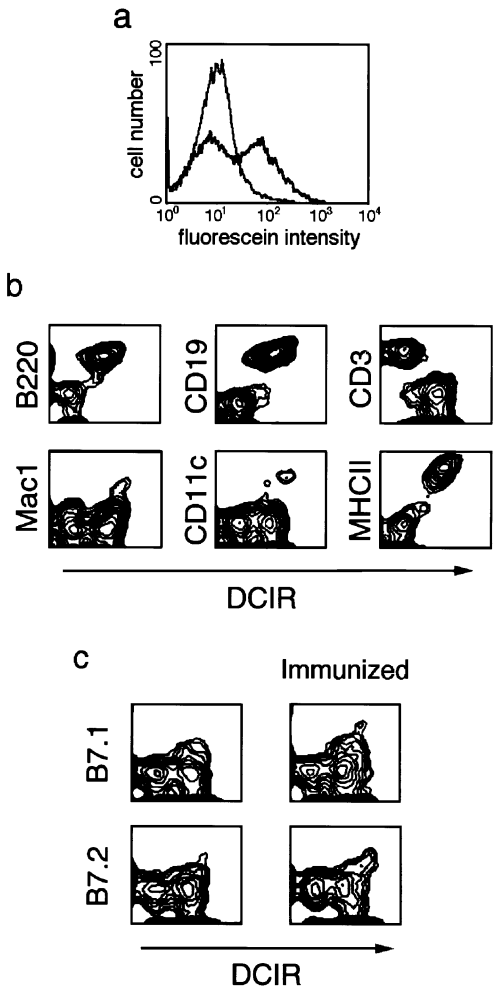

Figure 4. Mouse DCIR expression in splenic APC. (*a*) Collagenase-treated mouse splenic cells were stained with anti-DCIR antibodies (*thick line*) or the same amount of normal rabbit Ig (*thin line*). (*b*) Splenic cells were analyzed by two-color FCM using anti-DCIR antibodies and each leukocyte marker. (*c*) Flow diagram of DCIR and costimulatory molecules before (*left column*) and 7 d after (*right column*) intraperitoneal immunization with sheep red blood cells.

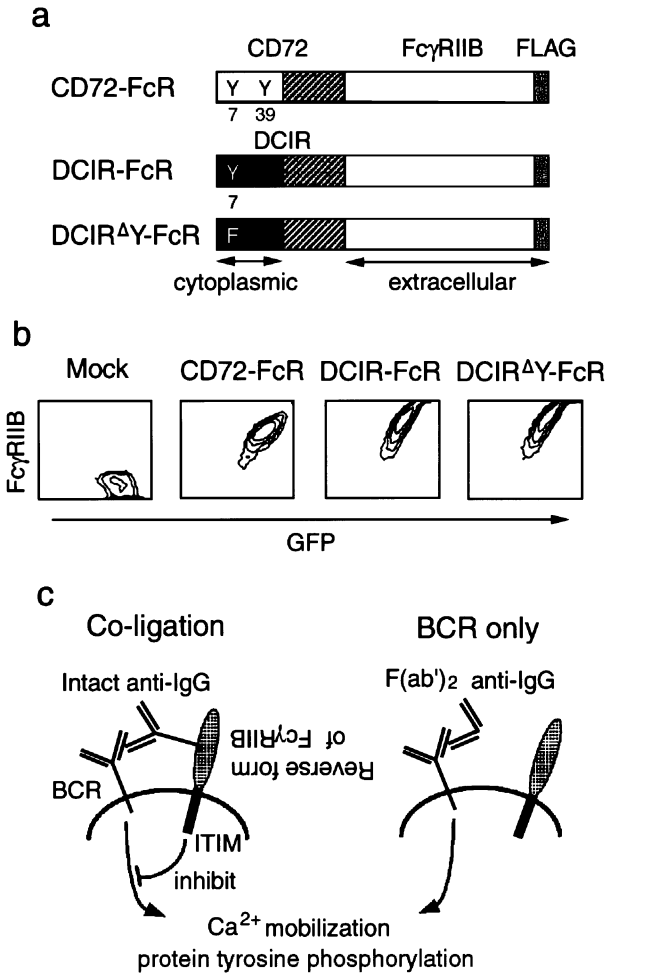

Figure 5. Production of IIA1.6 cells expressing chimeric FcR. (*a*) Diagram of constructs: cytoplasmic and transmembrane domain of mouse CD72, DCIR, and its $^{\Delta}$Y mutant were fused with extracellular FcγRIIB containing FLAG epitope in its C-terminus. (*b*) Cell surface expression of chimeric FcR. Sorted GFP-positive transformants were analyzed by two-color FCM using anti-TCR γδ. (*c*) A schematic view of stimulation. Incubation with intact antimouse IgG results in coligation of BCR and chimeric FcR (*left*), whereas its $F(ab')_2$ fragment results in ligation of BCR only (*right*).

DCIR engagement inhibits B cell receptor (BCR)-mediated Ca^{2+} mobilization depending on its ITIM tyrosine To reveal the inhibitory function of DCIR in APC, its engagement in BCR-mediated activation signal was attempted. For this purpose, a chimeric receptor containing cytoplasmic mouse DCIR and extracellular FcγRIIB tagged by FLAG epitope in its C-terminus (DCIR-FcR) was constructed and introduced into IIA1.6 cells using the retroviral pMX-IRES-EGFP vector. IIA1.6 is a variant of A20/2 J mouse B cell lymphoma lacking cell surface Fc receptors (Jones *et al*, 1986). As illustrated in **Fig 5**(***a***), two other chimeric Fc receptors were constructed and transfected: mutant DCIR-FcR (DCIR$^{\Delta}$Y-FcR), whose ITIM tyrosine at position 7 was replaced with phenylalanine, and CD72-FcR including cytoplasmic CD72, a well-investigated ITIM-bearing type II lectin receptor that can negatively regulate signaling through BCR (Adachi *et al*, 2000). Notably, as FcγRIIB is a type I membrane protein, the N-terminus of extracellular FcγRIIB was close to the plasma membrane and it stood reverse to its natural form. As shown in **Fig 5**(***b***), however, sorted GFP-positive transformants were positively stained by anti-γδ TCR monoclonal antibody, whereas mock-transfected IIA1.6 cells of B cell origin were negatively stained, indicating that the reverse form of extracellular FcγRIIB can be recognized by the Fc portion of this antibody. The expression level of each chimeric FcR on the surface of these transformants was almost the same among these cells (**Fig 5***b*). These transformants were stimulated using $F(ab')_2$ or intact antimouse IgG antibodies, resulting in ligation of BCR only or coligation of BCR and chimeric FcR, respectively, as illustrated in **Fig 5**(***c***).

We first examined the effect of DCIR engagement in BCR-mediated Ca^{2+} mobilization using fura-red as an indicator. After ligation of BCR only, each transformant showed an immediate decrease and gradual recovery of cellular fura-red (**Fig 6,** Mock and BCR only), indicating transient rapid increase of intracellular Ca^{2+} concentration (Burchiel *et al*, 2000). This Ca^{2+} mobilization was fully inhibited when CD72-FcR was coligated with BCR (**Fig 6,** CD72-FcR), indicating that cytoplasmic CD72 as a positive control can deliver a negative regulatory signal using this system. DCIR-FcR absolutely blocked the Ca^{2+} mobilization on coligation with BCR to the same degree as CD72-FcR (**Fig 6,** DCIR-FcR). These results reveal that engagement of cytoplasmic DCIR can inhibit BCR-mediated Ca^{2+} mobilization, at least one aspect of B cell activation, as is the case with cytoplasmic CD72. Furthermore, this inhibition was completely lost when the ITIM

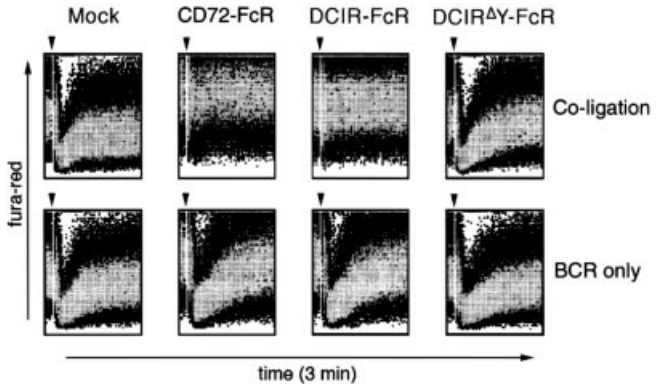

Figure 6. ITIM-dependent inhibition of BCR-mediated Ca^{2+} mobilization by coligation of DCIR-FcR and BCR. After stimulation, change of fura-red level loaded in each transformant was measured by FCM. *Arrowheads* indicate the timepoints of adding antimouse IgG.

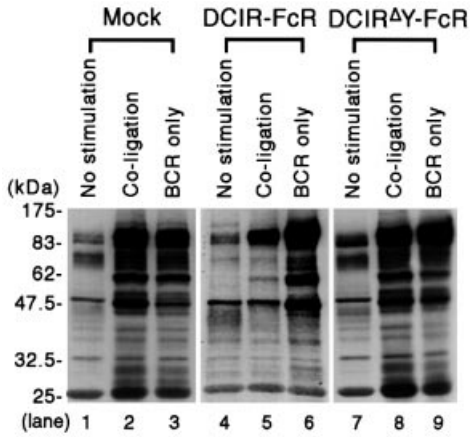

Figure 7. ITIM-dependent inhibition of BCR-mediated protein tyrosine phosphorylation by coligation of DCIR-FcR and BCR. After stimulation, each transformant was lysed and immunoprecipitated using anti-PY. Precipitates were analyzed by Western blotting using anti-PY.

tyrosine of DCIR was replaced with phenylalanine (**Fig 6,** $DCIR^{\Delta}Y$-FcR), indicating that the Ca^{2+}-mobilization-inhibiting effect of cytoplasmic DCIR is fully dependent on its ITIM tyrosine.

DCIR engagement inhibits BCR-mediated protein tyrosine phosphorylation depending on its ITIM tyrosine As it is known that BCR-mediated signal activates some kinase cascades (Kurosaki, 1998; Tamir and Cambier, 1998), we further examined the effect of DCIR engagement in BCR-mediated tyrosine phosphorylation of various proteins with immunoprecipitation and detection using anti-PY antibody. In **Fig 7** (Mock), more bands were visualized in *lanes 2* and *3* than in *lane 1* and they showed a higher intensity, indicating that cellular tyrosine-phosphorylated proteins increase both in number and in PY level when only BCR is ligated in mock-transformants. In **Fig 7** (DCIR-FcR), almost the same number of bands were visualized in *lane 5* as in *lane 4* and they did not show much higher intensity except for the 60 and 100 kDa bands, whereas in *lane 6* there were more bands and the bands were of higher intensity to the same degree as those in *lanes 2* and *3*. These results indicate that BCR-mediated protein tyrosine phosphorylation is remarkably inhibited when DCIR-FcR is coligated with BCR. In contrast, in **Fig 7** ($DCIR^{\Delta}Y$-FcR), again more bands were visualized in *lane 8* and they showed a higher intensity than those in *lane 7*, to the same degree as those in *lane 9*, indicating that BCR-mediated protein tyrosine phosphorylation is fully recovered when the ITIM tyrosine of DCIR is replaced with phenylalanine. These results demonstrate that engagement of cytoplasmic DCIR can inhibit BCR-mediated protein tyrosine phosphorylation depending on its ITIM tyrosine, as is the case with inhibition of Ca^{2+} mobilization, revealing another inhibitory effect of cytoplasmic DCIR through its ITIM.

DISCUSSION

In this study, we isolated mouse DCIR from BM-DC using the SST method. DCIR is a type II membrane protein bearing a single transmembrane domain near its N-terminus. The original SST method using COS cells was reported to be useful for isolation of secreted or type I membrane proteins bearing typical eukaryotic signal sequences (Tashiro *et al*, 1993). As a result of the yeast-using SST screening, however, which we performed in this study, about 10% of the clones obtained were type II proteins (Jacobs *et al*, 1997). The transmembrane region of DCIR, which contains more amino acid residues and shows higher hydrophobicity than a typical eukaryotic signal sequence, is considered to work as the secretory signal sequence in this yeast-SST system. As human and mouse DCIR were originally identified by the database search (Bates *et al*, 1999), it should be noted that the DCIR was actually isolated from the DC cDNA library. Recently, Bonkobara *et al* applied the original SST method to isolation of novel proteins from keratinocytes and reported some skin-specific molecules.[3] These results indicate that there still remain important molecules to be identified by the SST method in the dermatologic area as well as in immunology.

We detected expression of mouse DCIR in spleen and lymph node among normal tissues and splenic MHC class II^{+} APC including DC, B cells, and monocytes/macrophages, but not in cultured NK cells or splenic T cells (**Figs 1*a*, 2*b*, 4*b***). These results are quite consistent with those of the human counterpart (Bates *et al*, 1999). In addition, we observed a slight increase of mouse DCIR expression during *in vitro* culture of bone marrow cells with GM-CSF, suggesting a slight increase of its expression with DC maturation (**Fig 1*b***). This observation is not necessarily consistent with the previous report describing that expression of human DCIR was downregulated with *in vitro* DC terminal maturation induced by short-term culture with lipopolysaccharide or CD40 ligand after 7 d culture of blood monocytes with GM-CSF and IL-4 (Bates *et al*, 1999). It remains to be further investigated whether this discordance is due to the difference in species or the difference in the method of induction of DC maturation, but the result of FCM analysis showing that both mouse splenic $B7.1^{high}$ and $B7.2^{high}$ cells retained a strong expression of DCIR after *in vivo* immunization (**Fig 4*c***) supports our *in vitro* observation.

Most interestingly, our results presented some lines of evidence of the inhibitory DCIR function using a chimeric receptor composed of cytoplasmic mouse DCIR and extracellular FcγRIIB. FcγRIIB is one of the ITIM-bearing inhibitory receptors of the Ig superfamily and has been examined intensively (Coggeshall, 1998). When IgG is sufficiently produced to form immune complexes, FcγRIIB is coligated with BCR by this complex and downmodulates the BCR-mediated activation signal, resulting in negative feedback regulation of IgG production. This *in vivo* phenomenon is reproduced *in vitro* by coligation of BCR and FcγRIIB using anti-IgG antibody. Therefore, to investigate the intracellular signaling pathway through other ITIM-bearing Ig superfamily receptors, such as killer cell inhibitory receptors (Ono *et al*, 1997; Bruhns *et al*, 1999) and PD-1,[2] BCR-mediated signals after coligation with a chimeric FcγRIIB containing their cytoplasmic tail have been examined. In contrast, this study is the

[3]Bonkobara M, Das A, Shikano S, Cruz P Jr, Ariizumi K: Effective use of a signal-sequence-trap method to identify novel proteins secreted by keratinocytes. *J Invest Dermatol* 114:806, 2000 (abstr.)

first trial to coligate BCR with a chimeric receptor of cytoplasmic ITIM-bearing type II receptor and extracellular FcγRIIB, in order to reveal the inhibitory function of the type II receptor. As shown in the results, the reverse form of extracellular FcγRIIB still works as an Fc receptor (**Fig 5*b***) and this system is useful to detect an inhibitory effect delivered by a type II ITIM-bearing receptor such as CD72 (**Fig 6**).

Using this method, we successfully showed a distinct inhibitory effect of DCIR: DCIR engagement can downregulate BCR-mediated Ca^{2+} mobilization as can CD72, and this effect requires its ITIM tyrosine residue (**Fig 6**). Furthermore, we revealed that DCIR engagement can downregulate BCR-mediated protein tyrosine phosphorylation depending on its ITIM tyrosine residue (**Fig 7**). This result reveals the second ITIM-dependent inhibitory effect of DCIR and suggests involvement of some phosphatase in DCIR-mediated inhibitory signal. The molecular mechanism of inhibitory signal mediated by mouse DCIR, including phosphorylation of ITIM tyrosine and phosphatase recruitment, is now under investigation. In a very recent report, human DCIR has been shown to bind SH2-domain-containing protein tyrosine phosphatase 1 in myeloid HL-60 cells after pervanadate treatment (Huang *et al*, 2001). Although the function of DCIR in the animal is still unknown, the results we have presented in this study show unexceptional expression of mouse DCIR in MHC class II^+ APC (**Fig 4*b***) and its distinct downregulating capacity of B cell activation signals (**Figs 6**, **7**). It remains to be further investigated whether DCIR can inhibit APC activation induced by anti-MHC class II antibody as is the case with immunoglobulin-like transcript 3 (Cella *et al*, 1997), and whether DCIR regulates apoptosis like CD72 and FcγRIIB (Ashman *et al*, 1996; Nomura *et al*, 1996). Even its potential relevance to the pathophysiology of atopy or allergic reactions should be proposed like other inhibitory receptors (Scharenberg, 1999). Discovery of natural ligands or coreceptors of DCIR, and phenotypic analysis of DCIR knockout mice or APC-specific transgenic mice, may reveal its function *in vivo*.

Recombinant mouse GM-CSF was kindly provided by Kirin Brewery Co. (Maebashi, Gunma, Japan). We would like to thank Professor Tasuku Honjo for fruitful discussions and continuous encouragement. We thank Ms. Naoko Tomikawa and Ms. Masami Tanaka for their technical assistance.

REFERENCES

Adachi T, Wakabayashi C, Nakayama T, Yakura H, Tsubata T: CD72 negatively regulates signaling through the antigen receptor of B cells. *J Immunol* 164:1223–1229, 2000

Ariizumi K, Shen GL, Shikano S, *et al:* Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J Biol Chem* 275:11957–11963, 2000a

Ariizumi K, Shen GL, Shikano S, *et al:* Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J Biol Chem* 275:20157–20167, 2000b

Ashman RF, Peckham D, Stunz LL: Fc receptor off-signal in the B cell involves apoptosis. *J Immunol* 157:5–11, 1996

Balch SG, McKnight AJ, Seldin MF, Gordon S: Cloning of a novel C-type lectin expressed by murine macrophages. *J Biol Chem* 273:18656–18664, 1998

Banchereau J, Steinman RM: Dendritic cells and the control of immunity. *Nature* 392:245–252, 1998

Bates EE, Fournier N, Garcia E, *et al:* APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif. *J Immunol* 163:1973–1983, 1999

Bolland S, Ravetch JV: Inhibitory pathways triggered by ITIM-containing receptors. *Adv Immunol* 72:149–177, 1999

Bruhns P, Marchetti P, Fridman WH, Vivier E, Daeron M: Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. *J Immunol* 162:3168–3175, 1999

Burchiel SW, Edwards BS, Kuckuck FW, Lauer FT, Prossnitz ER, Ransom JT, Sklar LA: Analysis of free intracellular calcium by flow cytometry: multiparameter and pharmacologic applications. *Methods* 21:221–230, 2000

Cella M, Dohring C, Samaridis J, Dessing M, Brockhaus M, Lanzavecchia A, Colonna M: A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. *J Exp Med* 185:1743–1751, 1997

Coggeshall KM: Inhibitory signaling by B cell Fc gamma RIIb. *Curr Opin Immunol* 10:306–312, 1998

Crowley M, Inaba K, Witmer-Pack M, Steinman RM: The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* 118:108–125, 1989

Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, Figdor CG: Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100:575–585, 2000

Huang X, Yuan Z, Chen G, Zhang M, Zhang W, Yu Y, Cao X: Cloning and characterization of a novel ITIM containing lectin-like immunoreceptor LLIR and its two transmembrane region deletion variants. *Biochem Biophys Res Commun* 281:131–140, 2001

Inaba K, Inaba M, Romani N, *et al:* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–1702, 1992

Jacobs KA, Collins-Racie LA, Colbert M, *et al:* A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* 198:289–296, 1997

Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC: The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* 375:151–155, 1995

Jones B, Tite JP, Janeway CA: Different phenotypic variants of the mouse B cell tumor A20/2J are selected by antigen- and mitogen-triggered cytotoxicity of L3T4-positive, I-A-restricted T cell clones. *J Immunol* 136:348–356, 1986

Kanazawa N, Nakamura T, Tashiro K, *et al:* Fractalkine and macrophage-derived chemokine: T cell-attracting chemokines expressed in T cell area dendritic cells. *Eur J Immunol* 29:1925–1932, 1999

Kitamura T: New experimental approaches in retrovirus-mediated expression screening. *Int J Hematol* 67:351–359, 1998

Kurosaki T: Molecular dissection of B cell antigen receptor signaling. *Int J Mol Med* 1:515–527, 1998

Matsumoto M, Tanaka T, Kaisho T, *et al:* A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. *J Immunol* 163:5039–5048, 1999

Mizuochi S, Akimoto Y, Imai Y, Hirano H, Irimura T: Unique tissue distribution of a mouse macrophage C-type lectin. *Glycobiology* 7:137–146, 1997

Nomura T, Han H, Howard MC, Yagita H, Yakura H, Honjo T, Tsubata T: Antigen receptor-mediated B cell death is blocked by signaling via CD72 or treatment with dextran sulfate and is defective in autoimmunity-prone mice. *Int Immunol* 8:867–875, 1996

Numa F, Hirabayashi K, Tsunaga N, *et al:* Elevated levels of syndecan-1 expression confer potent serum-dependent growth in human 293T cells. *Cancer Res* 55:4676–4680, 1995

Ogasawara K, Hida S, Azimi N, *et al:* Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature* 391:700–703, 1998

Ono M, Okada H, Bolland S, Yanagi S, Kurosaki T, Ravetch JV: Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell* 90:293–301, 1997

Scharenberg AM: The inhibitory receptor superfamily: potential relevance to atopy. *Curr Opin Immunol* 11:621–625, 1999

Soilleux EJ, Barten R, Trowsdale J: DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13. *J Immunol* 165:2937–2942, 2000

Stahl PD, Ezekowitz RA: The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol* 10:50–55, 1998

Tamir I, Cambier JC: Antigen receptor signaling: integration of protein tyrosine kinase functions. *Oncogene* 17:1353–1364, 1998

Tashiro K, Tada H, Heilker R, Shirozu M, Nakano T, Honjo T: Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* 261:600–603, 1993

Umesono K, Murakami KK, Thompson CC, Evans RM: Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* 65:1255–1266, 1991

Valladeau J, Ravel O, Dezutter-Dambuyant C, *et al:* Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 12:71–81, 2000

Vivier E, Daeron M: Immunoreceptor tyrosine-based inhibition motifs. *Immunol Today* 18:286–291, 1997

Yabe D, Nakamura T, Kanazawa N, Tashiro K, Honjo T: Calumenin a Ca^{2+}-binding protein retained in the endoplasmic reticulum with a novel carboxyl-terminal sequence, HDEF. *J Biol Chem* 272:18232–18239, 1997